Sustainable Bio-fabrication of Silver Nanoparticles via *Terminalia bellerica* Leaf Extract to Enhance Antioxidant and Antimicrobial Efficacy

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Abstract

Nanoparticles have become significant in biomedical fields due to its angiogenesis-inhibiting, antimicrobial, antiviral and anti-inflammatory properties. This study involved producing a straightforward, eco-friendly reliable and cost-efficient technique for producing silver nanoparticles (Ag-NPs) by leaf extracts of *Terminalia bellerica*. $AgNO_3$ solution and an aqueous extract of *Terminalia bellerica* were used in a reduction process to make Ag-NPs. The characterization was performed through visual inspection, UV–Vis spectroscopy, and Fourier-transform infrared spectroscopy. Experimental outcome revealed that *Terminalia bellerica* leaf extract mediated Ag-NPs exhibited a yellowish to brown color with maximum absorption peak of at 460 nm, confirming the successful bio-fabrication of Ag-NPs. Furthermore Ag-NPs remained stable at 5°C for up to 15 days. According to optimization results, the production of Ag-NPs is maximum at a pH of 11, with incubation temperature of 80°C, and a concentration is 1 mM $AgNO_3$, which ensures stability and prevents aggregation. These conditions promote the reduction of Ag+ ions for effective synthesis of Ag-NPs, resulting in a high yield and reliable quality for practical applications. These Ag-NPs demonstrated strong antimicrobial activity against *Staphylococcus aureus* (MTCC-96), *Pseudomonas aeruginosa* (MTCC-1688), *Escherichia coli* (MTCC-1302), *Bacillus cereus* (MTCC-1307) and *Salmonella Typhi* (MTCC-98), found minimum inhibitory concentration lies between from 0.5 µg/ml to 5 µg/ml. This study came to the conclusion that leaf extract of *Terminalia bellerica* is a useful reducing agent for creating stable silver nanoparticles with strong antioxidant and antibacterial properties.

Keywords: *Terminalia bellerica, Silver Nanoparticles, Optimization, Antioxidant, Minimum inhibitory concentration.*

Introduction

Nanotechnology is the development of small particles, ranging from 1 to 100 nm, with unique characteristics that are useful in the fields of medicine, renewable resources, and health. Important properties of metal nanoparticles include their high surface-to-volume ratio, controlled synthesis, strong optoelectronic, thermal, and catalytic efficiency. Particularly useful metals for creating nanoparticles include zinc, silver, iron, copper oxide, gold, and platinum [1]. Ag-NPs are gaining attention in medicine for their antimicrobial, anticancer, and antioxidant properties. Green synthesis using medicinal plant extracts offers a sustainable, low-toxicity method with high yields and energy efficiency. In this process, bioactive compounds in plant extracts reduce Ag^{+} to Ag^{0}, eliminating the need for toxic chemicals [2]. Ag-NPs exhibit broad-spectrum antimicrobial effects and selective anticancer activity [3]. Ag-NPs can be produced by using mainly two methods: "top-down" and "bottom-up". Top-down technique begins with a bulk material, which is mechanically broken into nanoscale particles [4]. However, achieving a narrow size range is challenging by this approach. The bottom-up method, starting with atomic-level materials, uses chemical techniques like hydrothermal, sol-gel, gas-phase, or hydrolysis methods to build nanoparticles. Although these methods provide more control to regulate the shape and particle size, surface chemistry can be harder to manage. Overall, bottom-up approach is preferred due to better control over particle formation. Ag-NPs may produce by using physical methods, chemical methods, and biological approaches [5]. These physical and chemical approaches have drawbacks, such as long preparation times, high costs, energy consumption, and environmental concerns. Physical methods like milling, gas-phase deposition, laser ablation, and produce high-purity nanoparticles but result in larger sizes compared to chemical or biological methods. Chemical methods require stabilizers and reducing agents (e.g. citric acid, hydrazine, formic acid, sodium citrate, sodium borohydride) to prevent aggregation and reduce Ag^{+} to Ag^{0} [6]. Biological approaches, using agents like plant-mediated synthesis, algae-mediated synthesis, microbial-mediated synthesis, biopolymer-assisted synthesis and enzyme-mediated synthesis [7,8]. Using plants and their extracts to synthesize metal nanoparticles offers advantages due to their abundance, safety, and diverse metabolites, which aid in the reduction process. In the formation of nanoparticles, plant extracts can have two functions they can stabilize the formed nanoparticles and reduce metal ions. These extracts contain natural compounds like polyphenols, flavonoids, polysaccharides

and alkaloids, which help convert metal ions into their metallic form. Additionally, these compounds prevent the nanoparticles from clumping together, keeping them stable. This makes plant extracts a great, eco-friendly choice for producing nanoparticles in a sustainable way [9]. Size and antibacterial properties of the nanoparticles depend on the plant's phytochemical composition, concentration, synthesis temperature, and reaction time [10]. Several plants, including *Argemone maxicana*, *Syzygium cumuni*, *Cinnamomum zeylanicum*, *Ocimum tenuiflorum*, *Mukia maderaspatana* and *Acalypha indica*, have been used for synthesizing mineral nanoparticles [11]. The biosynthesis of Ag-NPs involves preparing a silver salt solution and plant extract by using an appropriate solvent at a specific temperature. The silver salt solution and extract are then mixed in varying proportions, adjusting pH, temperature, and time, where the phytochemical reduces Ag^{+} ions to Ag^{0}, forming aggregates that lead to nanoparticle formation. Afterward, the nanoparticles are purified through centrifugation to eliminate excess or unreacted plant extract, resuspended in distilled water and subjected to a second round of centrifugation to remove any unwanted substances. Water is preferred solvent for extracting biomolecules due to its polarity, easily to handle, and non-toxicity, although ethanol can also be used. Several factors including $AgNO_3$ concentration, type of plant extract, incubation time, plant extract to $AgNO_3$ solution ratio, pH, temperature, and influence the stability, size and shape of the formed nanoparticles, which affect their biological activity [2]. Finally, various analytical methods such as UV–visible spectrophotometry, X-ray diffraction, and transmission electron microscopy, field emission scanning electron microscope, fourier transform infrared spectroscopy and energy dispersive X-ray spectroscopy are used to check the formation of Ag-NPs [9]. Forthcoming research must be emphasis on optimizing synthesis, exploring new plant sources, and evaluating the ecofriendly and health benefits of Ag-NPs. However, challenges like extract variability, scalability, stability, and toxicity need to be addressed.

This study mainly emphases on an environmentally friendly, sustainable, and cost-efficient green synthesis of Ag-NPs from *Terminalia bellerica* (*T. bellerica*) leaf extract containing various phytochemicals like amines, alkaloids, terpenes, flavonoids, and tannins. *T. bellerica* extract was used to produce Ag-NPs to investigate antimicrobial and antioxidant activity. It also looks at the influence of temperature, pH, the ratio of silver nitrate to extract, reaction time, extract type, and on the synthesis of nanoparticles, antimicrobial and antioxidant potential.

Terminalia bellerica is a major ingredient in the Ayurvedic formula "Triphala," which has long been used to support longevity, immunity, and health [12]. The leaves have a lot of therapeutic potential even though the fruits have been studied extensively. Rich in antioxidants, the leaves of *T. bellerica* may help treat a number of conditions, including chronic ulcers, fever, jaundice, constipation, asthma, and anemia [13]. The plant contains a number of bioactive substances, such as chebulagic acid, beta-sitosterol, ellagic acid, gallic acid, ethyl gallate, and different sugars and polyphenols, according to phytochemical studies. The current study intends to assess and contrast the antibacterial and antioxidant properties of *T. bellerica* leaf ethanolic extracts and active fractions in light of these findings, thereby emphasizing their potential for therapeutic use [14].

Method and material

Plant Material Preparation

The leaves of *T. bellerica* were freshly harvested from Chandigarh, ensuring they were both disease-free and of superior quality. To remove any dirt, dust, or potential contaminants, the leaves were continuously cleaned 1-2 times with running tap water. This thorough cleaning process was crucial to ensure the leaves were free from external impurities before proceeding with further use or processing. Using a pestle and mortar, the leaves were crushed into a powder and kept in a sealed container. Methanolic and aqueous extract was prepared by cold percolation method and then passed through the Whatman filter paper to remove any suspended material. The stock solution of different plant extracts was prepared meticulously, using 12% dimethyl sulfoxide, a universal solvent, to achieve a final 100mg/ml concentration. The extracts were thoroughly mixed and kept at 277 K in the refrigerator for further experiments.

Phytochemical screening of *T. bellerica* leaf extract

The aqueous and methanolic leaf extracts underwent preliminary phytochemical analysis following established protocols [15]. The analysis aimed to detect the occurrence or absence of various bioactive compounds in samples. The methods employed for the qualitative phytochemical screening tests are provided in the accompanying (Table 1). Phytochemical screening of plant extracts identifies key natural compounds including tannins, flavonoids, steroids, carbohydrates, alkaloids, glycosides, terpenoids, phenols, and saponins.

Table 1: Phytochemical test for *T. bellerica* leaf extract

Compound Name	Reagent/Test	Reference
Phenols	$FeCl_3$ test: 0.5 ml sample was mixed in 2 mL of 10% $FeCl_3$ solution. The appearance of a black or blue color signifies the occurrence of phenols or tannins.	[16]
Flavonoids	Alkaline reagent test: 3 mL sample was added with 10% NaOH. The addition of dilute acetic acid causes a yellow solution to turn colorless, confirming flavonoids.	[17]
Saponins	Frothing test: A 0.3 mL aliquot of the sample treated with 4 mL of Deionized water and heated upto boiling. Formation of frothing indicates the occurrence of saponins.	[18]
Alkaloids	Mayer's reagent test: 2 mL of the extract and 2mL of 1% HCl were mixed and keep in water bath for half hr. A greenish, creamy precipitate formed when Mayer's reagent was added dropwise to the cooled mixture after it had cooled to room temperature, confirming the presence of alkaloids.	[17]
Tannins	Lead acetate test: 2 mL of sample added with 4–5 drops of a 10% lead acetate solution. A white precipitate's development signifies the presence of tannins.	[19]

Terpenoids and Steroids	Few drops of the sample mixed with 3 mL of $CHCl_3$, then 2 mL of H_2SO_4 was added, resulting in the formation of distinct layers. The apperance of a red color at the interface shows the occurrence of terpenoids. A red coloration at the bottom layer suggests the occurrence of steroids.	[16]
Carbohydrates	Molisch's Test: 0.5 mL of alpha-naphthol to 3 mL of extract in alcohol, stir for 5 minutes, and then gradually pour 1 mL of strong acid along the walls of the test tube. Carbohydrates are showed by presence of violet color at the junction.	[20]

Determination of Total Phenolic content (TPC)

The TPC was estimated through the Folin-Ciocalteu reagent process. Gallic acid standards (5–100 µg/mL) were used to quantify the amount, and the following formula was used to express the TPC content as gallic acid equivalents (GAE) per gram of sample.

$$TPC(mg/g) = \frac{C \times V)}{M}$$

M = Mass of the extract (gram)

V= volume of sample (ml)

C= concentration of gallic acid (mg/ml).

Determination of Total Flavonoid Content (TFC)

The $AlCl_3$ method used to calculate the TFC in the *T. bellerica* leaf extract. A rutin standard curve (5-100 µg/ml) used to calculate the flavonoid concentration, which was then represented as rutin equivalents (RE) per gram of sample. The given formula was used to estimate the flavonoid content.

$$TFC\ (mg/g) = \frac{(C \times V)}{M}$$

V= extract volume (ml)

M= extract weight (gram)

C = rutin concentration (mg/ml) from the calibration curve.

Synthesis of Ag-NPs

$AgNO_3$ solution was reduced with *T. bellerica* extract for bio-fabrication of Ag-NPs. The procedure was started by combining 1 mL of the leaf extract with 100 mL of a 1 mM $AgNO_3$ solution, which was then left to incubate 30 °C in the dark for the entire night. UV spectrometer was used to monitor the reduction of Ag^+ ions. Following the reaction, the Ag-NPs were separated using centrifugation for ten minutes at 5000 rpm. Until the supernatant turned colorless, signifying the elimination of surplus silver ions, this procedure was repeated. For upcoming research, the resultant pellet was stored at dark and cold place.

Optimization of various parameters for Ag-NPs synthesis

The bio-fabrication of Ag-NPs was optimized through regulating various factors, including time, pH, temperature, $AgNO_3$ concentration, and plant extract concentration. The pH was tested at levels 3, 5, 7, 9, and 11, with adjustments made using 10% hydrochloric acid and 0.4% sodium hydroxide. Incubation time was observed throughout 0 to 1 hr to determine the optimal time for Ag-NPs production. The synthesis was also monitored at various temperatures (20 °C, 40 °C, 60 °C, and 80 °C) to find the best conditions. Several concentrations of $AgNO_3$ (0.5, 1.0, 1.5, and 2.0 mM) were tested to regulate the nanoparticle formation [11]. Additionally, extract to $AgNO_3$ concentration ratio was optimized through adjusting the amount of extract in a 1 mM $AgNO_3$ solution [1,21]. The production of nanoparticles was monitored by measuring their absorbance with UV-vis spectrophotometer within the range of 300–700 nm.

Stability Study

To evaluate their stability, optimized Ag-NPs solutions were stored in a dark environment for 20 days. After this period, the stability of Ag-NPs was evaluated through a UV-Vis spectral study. This method provided insight into any potential changes in the optical

properties of the Ag-NPs over time, such as shifts in the wavelength of maximum absorption or changes in absorption intensity. The stability of the nanoparticles could be efficiently determined by comparing the UV-Vis spectra obtained prior to and following storage. The stability of the nanoparticles could be effectively determined, ensuring their resilience and suitability for various applications [22].

Characterization of Ag-NPs

UV-Visible Spectrophotometry

In order to characterize the synthesized nanoparticles, the Ag-NPs solution was diluted with distilled water and the spectrum was measured within the 300-700 nm by using UV-Vis spectrophotometry. Within an hour of the reaction, silver ion reduction and the subsequent synthesis of Ag-NPs were observed. To account for any possible interference, $AgNO_3$ solution was used in a control experiment.

Fourier-transform infrared (FTIR) Spectroscopy

FTIR spectrometer was employed to study the functional groups on the nanoparticle surface. FTIR analysis, the prepared Ag-NPs solution was centrifuged at 10,000 rpm for 30 min. The resulting pellet was carefully separated and wash away 3 times with 10 mL of deionized water to remove any unbound components that were not associated with the Ag-NPs. The washed pellet was then dried using a vacuum drier to remove any residual moisture. Once dried, the pellet was analyzed using FT-IR spectroscopy to categorize the functional groups involved in the capping of the Ag-NPs. FT-IR spectra were recorded in the range of 4000-400 cm^{-1} to investigate the interactions involved between the extract components and the Ag-NPs [23].

Antimicrobial Activity

The antimicrobial potential of Ag-NPs was evaluated against Gram-negative bacteria (*P. aeruginosa* (MTCC-1688) *E. coli*; MTCC 1302) and *S. Typhi* (MTCC-98) as well as Gram-positive bacteria (*B. cereus* (MTCC-1307) and *S. aureus*; MTCC-96). The bacterial strains were cultured on nutrient agar, which contains peptone, beef extract, and NaCl. Before experimenting, the bacterial strains were inoculated into nutrient broth to promote their growth during the exponential phase.

Antibiotic used as Positive Controls

Gentamicin, an aminoglycoside antibiotic, is highly effective against aerobic bacteria. It works by binding irreversibly to the 30S ribosomal subunit, disrupting protein synthesis. This prevents proper mRNA translation, leading to the incorporation of incorrect amino acids and halting essential protein production, which results in bacterial cell death. Gentamicin is commonly used to treat severe infections, especially by antibiotic-resistant pathogenic bacteria [24].

Agar Well Diffusion Method

Ag-NPs were tested for their antimicrobial properties against clinical isolates. The plant extract diffuses into the agar medium, interacting with the test organism, and forming clear zones of inhibition across the wells after 24-48 hours of incubation at 37°C. The size of the inhibition zone is indicative of the extract's antimicrobial potency.

To perform the test sterile Muller-Hinton agar was used. A 20µl inoculum of the test organism was spread evenly on the plates. Five wells (6mm in diameter) made in the solidified agar by a sterile cork borer. Different volumes of each sample (20µl, 40µl, 60µl, and 80µl) were added to the wells in triplicates. The diameter of the inhibition zones around the wells was estimated to assess the antibacterial potential following a 24-48-hour incubation period at 37°C.

The Minimum Inhibitory Concentration (MIC)

The MIC was performed by using resazurin-based microtiter dilution method. Resazurin is a blue, non-fluorescent redox indicator that undergoes a color change to pink and exhibits fluorescence upon reduction by metabolically active microorganisms. The reduction of resazurin to its reduced form, resorufin, correlates with cellular metabolic activity, which is directly proportional to microbial growth. In the occurrence of an effective antimicrobial agent, microbial growth is inhibited, preventing the reduction of resazurin, thereby maintaining its blue color. This assay was performed in a microtiter. The first row of wells was filled with 100 µL of plant extract in 10% (v/v) DMSO in sterile water. Twofold serial dilutions of the extract were prepared across the plate by transferring 100 µL from one well to the next. Each well contain 100 µL of nutrient broth and 10 µL of resazurin solution. Subsequently, 10 µL of bacterial culture was added to each well. There were both positive and negative controls. For twenty-four hours, the plate was incubated at 37°C. The lowest concentration of plant extract at which a color

shift from purple to pink or colorless occurred, signifying inhibition of bacterial growth, was identified as the MIC.

Antioxidant Activity: Free Radical Scavenging Activity (RSA)

Antioxidant activity of Ag-NPs was assessed using (2,2-diphenyl-1-picrylhydrazyl) DPPH, a free radical that absorbs at 517 nm (purple), and ascorbic acid as a standard. When DPPH interacts with a substance that donates hydrogen, its violet color is eliminated, indicating a reduction in radical activity. A 0.1 mM DPPH solution was combined with samples at various concentrations. Absorbance at 517 nm was measured after 30 minutes in the dark at room temperature. The following formula was used to calculate the RSA.

$$\text{RSA}\ (\%) = \frac{(\text{Abs. control} - \text{Abs. of sample})}{\text{Abs. of control}} \times 100$$

Results And Discussion

Phytochemical analysis

The of the aqueous and methanolic extracts of *T. bellerica* was conducted to identify various constituents, including terpenes, tannins, phenols, flavonoids, carbohydrates, glycosides, steroids, saponins, and alkaloids (Table 2). The results showed that the aqueous extract of *T. bellerica* contained alkaloids, flavonoids, glycosides, steroids, and phenols. The methanolic extract, however, did not give satisfactory results. Therefore, we decided to proceed with the aqueous extract for further experiments.

Table 2: Phytochemical analysis of of *T. bellerica* leaf extract

Phytoconstituents	***T. bellerica* (aqueous extract)**	***T. bellerica* (methanolic extract)**
Tannins and Phenols	+	-
Flavonoids	+	+
Glycosides	+	+
Steroids	+	-
Saponins	-	+
Terpenes	-	-
Alkaloids	+	-
Carbohydrate	+	+

Quantitative Analysis of TPC and TFC

Phenolic compounds are recognized as very effective antioxidants, contributing significantly to the ability to neutralize radicals in both medical and food applications. Measuring TPC and TFC is essential for assessing the radical neutralizing potential of plant extracts. In this study, the TPC of aqueous extracts from the leaves of *T. bellerica* was estimated using a gallic acid standard curve while the total flavonoid content was quantified using a rutin standard curve. TPC and TFC were observed across the concentration range of 5-100 mg of extract. TPC was found 143 ± 0.59 mg/g GAE. Conversely, the sample contained total flavonoid content 65.79 ± 0.73 mg/g RE. In a previous study leaves of *T. bellerica* TFC (170.54±4.17 mg/g GAE) and leaves of *T. bellerica* TFC (93.17±8.77 mg/g RE reported by [25]. Similarly, Chandel also found parallel results in the ethanolic extract of *T. bellerica*, with TPC of 169 ± 0.39 mg/g (GAE) and TFC of 79.79 ± 0.73 mg/g (RE) [26].

Ag-NPs Synthesis

This study examined the use of an aqueous extract of *T. bellerica* as a natural reducing agent in the bio fabrication of Ag-NPs. The extract, which is well-known for its antimicrobial qualities, successfully transformed $AgNO_3$ into Ag-NPs. The reduction mechanism was visually confirmed by the color shift of the reaction solution from colorless to yellowish and finally brown, which indicated the synthesis of Ag-NPs. This color shift indicates the formation of Ag-NPs because the plant leaf extract reduces silver ions, which causes silver particles to nucleate and grow. The surface plasmon resonance (SPR) factor, which absorbs visible light, is what causes the color shift in Ag-NPs. This colour change is shown in (Figure 1), which also shows the visible signs of the synthesis of Ag-NPs in the reaction solutions. The colors of Ag-NPs vary from pale yellow to brown. Krithiga et al., also noted similar results [27].

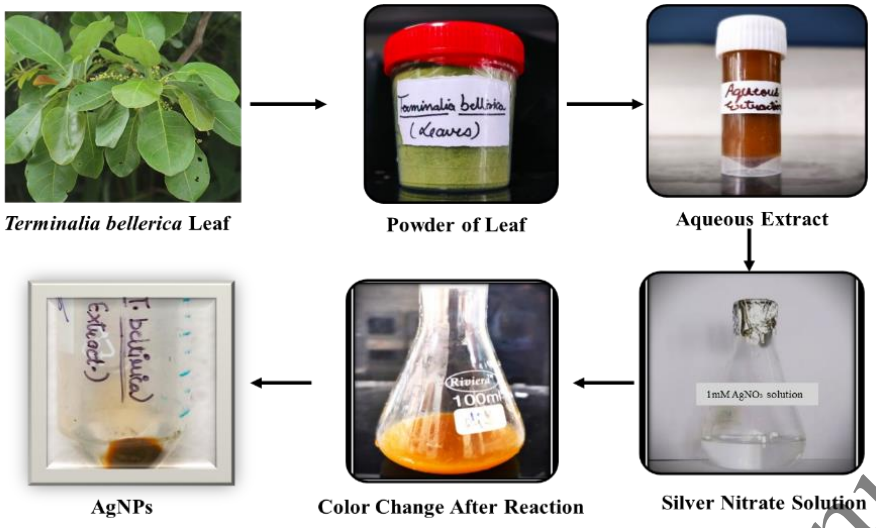

Figure 1: Green Bio-fabrication of Ag-NPs from *T. bellerica* leaf extract

Stability of Ag-NPs

The stability of bio-fabricated Ag-NPs was estimated by UV-Vis spectrometer after 20 days. Ag-NPs displayed a significant SPR peak at the wavelength (λ_{max}) with consistent absorption intensity. The absorbance of Ag-NPs may decrease with storage due to aggregation or oxidation. This observation confirmed the strong stability of the Ag-NPs over the 20 days, highlighting the effectiveness and convenience of this synthesis method for producing stable nanoparticles similarly Liaqat reported the same results [22].

Characterization of Ag-NPs

UV-Visible Spectrophotometry

UV-visible spectrometric analysis of the freshly prepared Ag-NPs confirmed this phenomenon. This color change is due to the phenomenon of SPR. The UV-visible spectroscopic analysis of synthesized Ag-NPs displayed a characteristic peak at 440-470 nm (Figure 2). Ag-NPs possess free electrons that resonate with incident light, resulting in the SPR absorption band. Likewise, Prasad and Elumalai observed similar absorption spectra of Ag-NPs solution showed a prominent absorbance in the 430–440 nm range [28]
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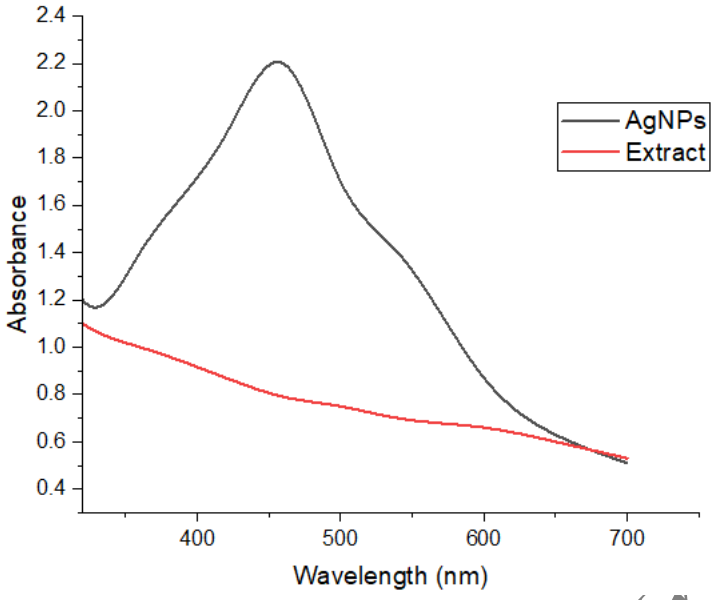


Figure 2: UV–Vis Spectra of Ag-NPs Synthesized via *T. bellerica* Leaf Extract-Mediated Reduction

Fourier-transform infrared (FT-IR) Spectroscopy

FT-IR spectrometry was used to characterize Ag-NPs made from T. bellerica leaf extract. Key peaks in the FTIR of Ag-NPs produced by T. bellerica indicated the presence of various functional groups, as seen in figure 3. O-H stretching is linked to the peaks at 3901.7 cm^{-1} and 3782.3 cm^{-1}, which indicate hydroxyl groups. Peaks between 3399.4 cm−1 indicated N-H stretching, pointing to amine or amide groups. The C=O peak at 1593.88 cm−1 indicated carbonyl groups. The peak at 573.5 cm^{-1} resembles to the C-I stretching, which is characteristic of alkyl and aryl halides, suggesting the participation of halogenated components in the formation of the Ag-NPs. The shift in the C=O peak seen in Ag-NPs' FTIR spectra raises the possibility that functional groups with carbonyls are essential to the bio reduction of silver ions to create Ag-NPs. Furthermore, the O-H and N-H stretching vibrations' downward shift suggests that these functional groups are probably interacting and binding with the Ag-NPs' surface to help stabilize them and stop them from aggregation [29]. Similarly, Prasad and Elumalai emphasized that (–C=O), (–OH), and (–NH) groups play a key role in the synthesis of Ag-NPs [28].

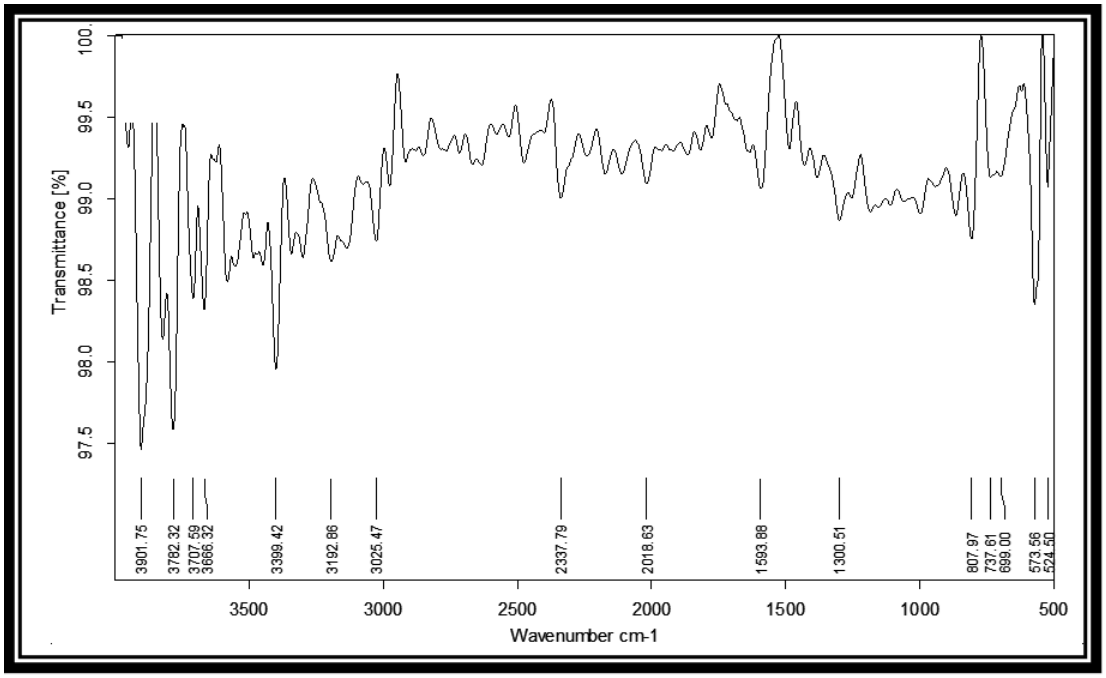


Figure 3: Fourier-Transform Infrared (FT-IR) Spectral Analysis of Ag-NPs

Optimization of Ag-NPs Synthesis

Optimization was conducted to increase the yield and stability of Ag-NPs. Key growth factors were systematically optimized, including the $AgNO_3$ concentration, pH, and temperature, which directly affect the production, size and stability of Ag-NPs.

Effect of $AgNO_3$ concentration

The primary factor influencing the production of Ag-NPs is the concentration of AgNO3. For effective production, yield maximization, and nanoparticle size control, this concentration must be optimized. AgNO3 concentrations ranging from 0.1 to 5 mM were used to examine the impact of AgNO3 concentration on the production of Ag-NPs. UV-Vis spectral analysis was used to observe the synthesis of Ag-NPs (figure 4), and it showed that a complete reduction of Ag+ occurred when the concentration of AgNO3 was increased up to 1 mM. However, excessively high concentrations can lead to particle aggregation and instability, highlighting the need for careful balance to optimize the synthesis process and ensure the desired nanoparticle characteristics. Our findings align with few past study of Saxena et al., which observed the highest synthesis of Ag-NPs at 2 mM $AgNO_3$ concentration [7]

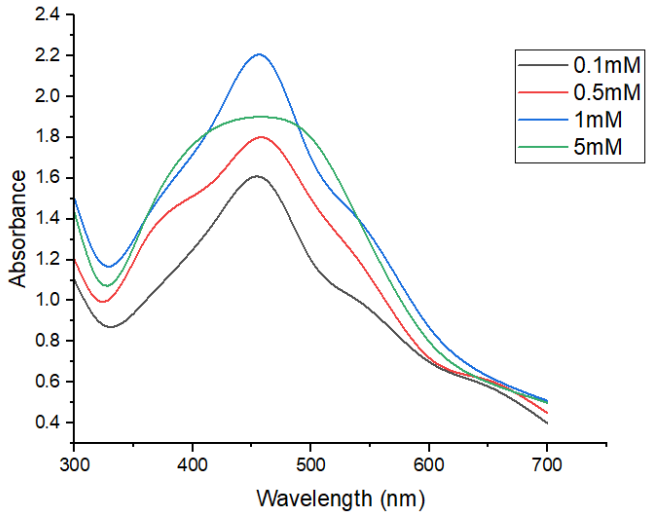


Figure 4: Effect of $AgNO_3$ Concentration on the UV–Vis Spectra of Synthesized Ag-NPs

Effect of pH

pH is a critical factor influencing the production of Ag-NPs. To determine the optimal pH for maximum nanoparticle synthesis, at different pH levels (4, 7, 9, and 11). The highest production of Ag-NPs was observed at pH 11, as indicated by a noticeable color change, further confirmed by UV–visible absorption spectra when compared to other pH values (Figure 5). Similar results have been studied by Liaqat et al by using *Eucalyptus camaldulensis* and *Terminalia arjuna* extracts which support the present study[30].

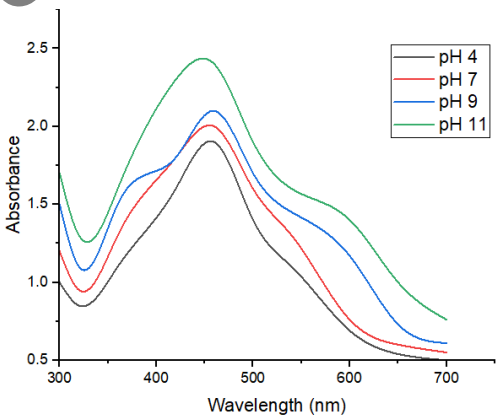


Figure 5: Effect of pH on the UV–Vis Spectra of Synthesized Silver Nanoparticles (Ag-NPs)

Effect of temperature

In order to examine the impact of incubation temperature on the production of Ag-NPs, 1 mM AgNO3 was added, and the mixture was incubated at 20°C to 80°C in increments of 20°C. Ag-NPs' productivity has been recorded over time. The highest production of Ag-NPs occurred at 80°C, where the nanoparticles remained stable for a prolonged period, indicating consistent synthesis (Figure 6). The increase in temperature enhances molecular kinetic energy, facilitating faster reduction of Ag^{+} ions and promoting more efficient nanoparticle formation, as previously reported by Birla [31].

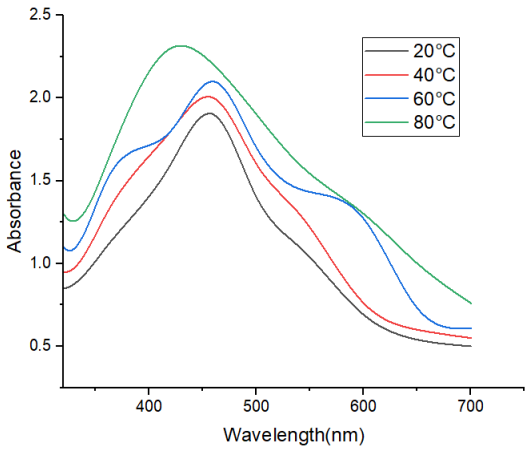
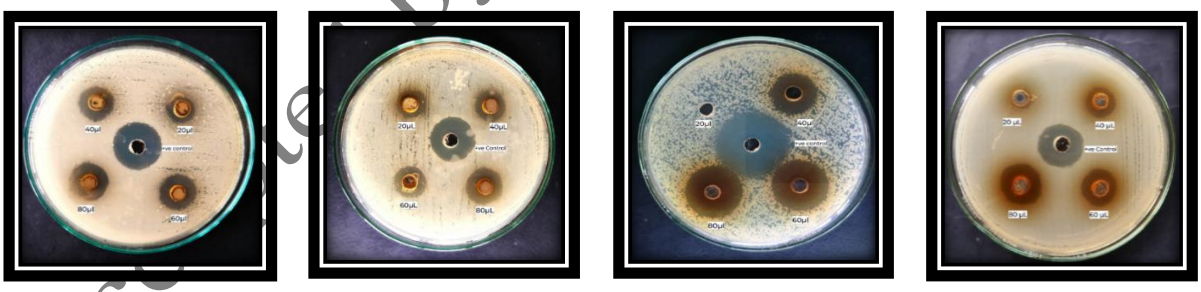


Figure 6: Effect of Incubation Temperature on UV–Vis Absorption Spectra of Synthesized Ag-NPs

Antibacterial Activity of Ag-NPs.

By employing the agar well diffusion method for determination of antimicrobial activity of prepared Ag-NPs, which were characterized from an aqueous extract of *T. bellerica*, was assessed against a range of pathogenic bacterial strains, including *S. typhi*, *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. cereus* (figure 7). The maximum volume tested was 80µl. The experimental results showed that the antimicrobial potential of the Ag-NPs was superior to that of the crude extract, exhibiting a more pronounced and larger zone of inhibition. The Ag-NPs displayed strong antibacterial potential towards Gram-positive bacteria (*S. aureus* 21±0.5mm and *B. cereus* 17±0.7mm) (Table 3). This finding aligns with previous research suggesting that Gram-negative bacteria's reduced susceptibility to Ag-NPs due to the presence of lipopolysaccharides (LPS) in the bacterial cell walls. The LPS molecules, which are negatively charged, can trap and block the positively charged Ag-NPs, preventing them from effectively interacting with the bacterial cell membrane as a result, Gram-negative bacteria exhibit a lower level of sensitivity to the bactericidal

effects of Ag-NPs compared to Gram-positive bacteria, which lack this protective outer layer and are more readily affected by the nanoparticles [32]. The significance of understanding the specific interactions between nanoparticles and bacterial cell structures to maximize their use in antimicrobial applications is underscored by this differential susceptibility. Ag-NPs block phosphate from entering bacterial cells and from being released from various metabolic intermediates, such as phosphates, mannitol, succinate, proline, and glutamine. This disturbance of phosphate homeostasis hampers essential cellular processes like energy production and macromolecule synthesis. The strong affinity of Ag-NPs for sulfur and phosphorus is central to their antibacterial properties [33]. Silver ions interact with sulfur-containing molecules (like proteins) and phosphorus-containing compounds (like nucleic acids and phospholipids), interfering with the structure and function of critical cellular components, thereby inhibiting bacterial growth and survival. The size of nanoparticles is crucial for antibacterial activity. Smaller Ag-NPs are readily absorbed by bacterial cell membranes, providing a bigger surface area for interaction. This makes it easier for Ag^{+} to be released through oxidation, which raises the generation of reactive oxidative species that harm cell structures and cause cell death. Additionally, silver atoms may combine with thiol groups in enzymes, forming stable S-Ag bonds, which deactivate enzymes and disrupt the bacterial cell membrane, causing cell lysis and contributing to the antibacterial effect [34].



B. subtilis ***P. aeruginosa*** ***S. aureus*** ***S. typhi***

Figure 7: Effect of Ag-NPs concentrations on the pathogenic bacterial strains

Table 3: Inhibition Zone Diameters (mm) Indicating Antibacterial potential of Green-Synthesized Ag-NPs

Concentration of Ag-NPs aqueous extract (µg/ml)	**ZOI (in mm) for the tested bacteria**				
	B. cereus	***P. aeruginosa***	***S. aureus***	***S. typhi***	***E. coli***

20	8±0.5	8±0.9	5±0.6	6±0.6	6±0.6
40	12±1	9±1	16±1.5	9±1	8±1
60	15±0.4	11±0.7	17±1	12±0.4	10±0.5
80	17±0.7	13±1	21±0.5	15±1.7	16±0.6
Gentamicin	24±0.8	23±0.6	27±1.6	24±1.4	25±1

Antibacterial Activity: Minimum Inhibitory Concentration of Ag-NPs

T. bellerica leaf extract mediated Ag-NPs MIC was assessed against various bacterial strains. The finding revealed that the MIC for *E. coli* was 5 mg/100µl, while for *S. typhi, B. cereus* and *S. aureus* it was 1.2 µg/mL. The MIC values for *S. flexneri* and *P. aeruginosa* were 2.5 µg/mL and 0.625 µg/mL respectively (Figure 8 and Table 4). These findings suggest that Ag-NPs were most effective against *B. cereus*, with the lowest MIC, and less effective against *E. coli*, requiring a higher concentration for inhibition. All values represent the mean of triplicate experiments, ensuring consistency and reliability of the results. Shoukani carried out a similar study where CuO-NPs were synthesized and established for their potential to inhibit bacterial growth these findings support the present study. The findings revealed that the CuO-NPs were effective at concentrations of 2.5 µg/mL towards bacteria like *S. aureus*, *E. faecalis*, and *S. typhi*. For other bacteria like *E. coli, K. pneumoniae*, and *P. aeruginosa*, a higher concentration of 5 µg/mL was needed to prevent their growth [35].

Figure 8: Microtiter plate showing MIC of Ag-NPs against various pathogenic bacterial strain

Table 4: MIC of Ag-NPs of *T. bellerica* against several pathogenic bacterial strains

Sr. No.	Concentration of Plant extract (µg/mL)	*E. coli*	*S. aureus*	*B. cereus*	*S. typhi*	*S. flexneri*	*P. aeruginosa*	*P. vulgaris*
1	10	-	-	-	-	-	-	-
2	5	-	-	-	-	-	-	-
3	2.5	+	-	-	-	-	-	-
4	1.2	+	-	-	-	+	-	-
5	0.625	+	+	+	+	+	-	-
6	0.3125	+	+	+	+	+	+	+
7	0.1562	+	+	+	+	+	+	+
8	0.0781	+	+	+	+	+	+	+
9	0.0395	+	+	+	+	+	+	+
10	0.01975	+	+	+	+	+	+	+
MIC (µg/mL)		5	1.2	1.2	1.2	2.5	0.625	0.1562

(-) = no bacterial growth; (+) = bacterial growth

Antioxidant Activity

The Ag-NPs produced from the *T. bellerica* exhibited significant free radical scavenging activity against DPPH, as illustrated in (figure 9). Notably, the Ag-NPs produced from *T. bellerica* displayed significant scavenging capacity, demonstrating substantial antioxidant activity when compared to ascorbic acid, a well-established antioxidant. The addition of an aqueous extract of *T. bellerica* to the DPPH solution resulted in a rapid decline in absorbance at 517 nm, indicating the strong scavenging ability of the Ag-NPs. The DPPH free RSA of Ag-NPs leaf extracts increase with concentration, indicating stronger antioxidant activity at higher levels. This reflects the effectiveness of the bioactive compounds in neutralizing free radicals, showcasing their potential as natural antioxidants for health and medicinal use. The antioxidant potential of Ag-NPs is mainly due to their unique physicochemical properties, enabling them to interact with and neutralize reactive oxygen species (ROS) and free radicals [30]. Ag-NPs can interact with radicals more effectively due to their grater surface area-to-volume ratio. By serving as electron donors, they stabilize ROS and stop oxidative damage. Ag-NPs' small size and specific shapes make them more reactive, and the additional antioxidant compound

produced during their synthesis from plant extracts enhances their scavenging ability. Ag-NPs also show SPR, which improves their ability to interact with ROS [36]. By scavenging free radicals, Ag-NPs help reduce oxidative stress, protecting against cellular damage linked to diseases, aging, and inflammation. Similar results reported by El-Rafie support the present study [37].

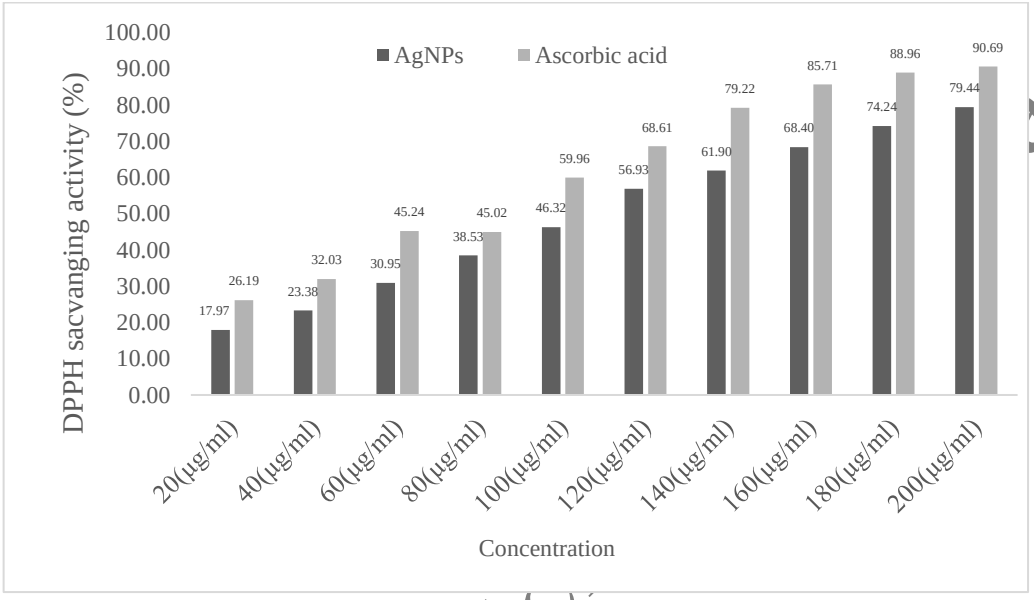


Figure 9: Comparative Analysis of DPPH Free RSA by Ag-NPs and Ascorbic Acid

Conclusion

The leaf extract of *T. bellerica* contains polyphenols and flavonoids that facilitate the synthesis of Ag-NPs. These Ag-NPs exhibited significant potential of antioxidant and antimicrobial. Phytochemical analysis of *T. bellerica* identified alkaloids, flavonoids, glycosides and steroids. The aqueous leaf extract contained 143 ± 0.59 mg/g of total phenolics (GAE) and 65.79 ± 0.73 mg/g of flavonoids (RE). Ag-NPs demonstrated stability at 5°C for up to 15 days. The optimal conditions for Ag-NPs production were identified as 1 mM $AgNO_3$ concentration, pH 11, and a temperature of 80°C, which ensured stability and effectively prevented aggregation. The aqueous extract of *T. bellerica* demonstrated notable antimicrobial activity, particularly against *P. aeruginosa*, *S. aureus*, *P. vulgaris S. typhi* and *B. subtilis*. The Ag-NPs produced from the extract showed enhanced antimicrobial efficacy, with the largest ZOI observed against *S. aureus* (23 mm). The MIC for the aqueous extract ranged from 2.5 µg/mL to 5 µg/mL for different bacterial strains. The MIC of Ag-NPs synthesized from *T. bellerica* aqueous extract was 5 mg/100µL for *E. coli*, 1.2 µg/mL for *S. typhi*, *B. cereus*, and *S. aureus*, 2.5

µg/mL for *S. flexneri*, and 0.625 µg/mL for *P. aeruginosa*. The extract also showed significant antioxidant activity at 20-200µg/ml.

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